Tuberculosis (2003) 83, 261–269

APPENDIX B

ELSEVIER

Tuberculosis

www.elsevierhealth.com/journals/tube

Resuscitation factors from mycobacteria: homologs of *Micrococcus luteus* proteins ☆

Wenming Zhu, Bonnie B. Plikaytis, Thomas M. Shinnick*

Tuberculosis/Mycobacteriology Branch, Division of AIDS, STD, and TB Laboratory Research, Centers for Disease Control and Prevention, 1600 Clifton Road NE, Atlanta, GA 30333, USA

KEYWORDS
Resuscitation factor;
Growth promotion;
Dormancy

Summary *Setting*: Resuscitation promoting factors (Rpfs) are proteins, originally identified in *Micrococcus luteus*, that promote recovery of bacteria from a viable but non-replicating phase (e.g., stationary phase or latency) to a replicating phase. Purified *M. luteus* Rpf can stimulate growth and increase recovery of *M. luteus* bacteria as well as *Mycobacterium tuberculosis* bacteria from prolonged stationary cultures.

Objective: To clone and characterize Rpfs from mycobacteria.

Design: We cloned one *M. avium* subsp. *paratuberculosis rpf* gene and one *M. tuberculosis rpf* gene into the pET19b or pET21a vector for expression in *Escherichia coli*. The His–tag recombinant proteins were purified and characterized.

Results: When the purified recombinant proteins were added to Sauton medium (a relatively minimal medium) at 100–500 pM, lag phase for mycobacteria from non-replicating cultures was shortened and there was a 10- to 100-fold increase in colony-forming units compared with control samples. In most probable number assays, the mycobacterial Rpfs increased recovery of mycobacteria from late stationary culture by about 10-fold. The Rpfs also promoted recovery of extensively washed *Mycobacterium smegmatis* bacteria inoculated into Sauton medium. Rpfs had only minor effects on growth of *M. tuberculosis* in BACTEC 12B broth, a rich medium.

Conclusion: The mycobacterial Rpfs demonstrate resuscitation activities similar to those of the *M. luteus* Rpf.

Published by Elsevier Science Ltd.

Introduction

Mycobacteria are important human and animal pathogens and cause a broad range of diseases from superficial skin infections to the devastating diseases of tuberculosis and leprosy. Among infectious diseases, tuberculosis (TB) remains the second leading killer of adults in the world, with more than 2 million TB-related deaths each year and more than 8 million new cases.[1,2] *Mycobacterium avium* subsp. *paratuberculosis* is the causative agent of Johne's disease in ruminants.[3] This mycobacterium has also been sporadically isolated from patients affected with Crohn's disease (CD).[4,5] CD is a chronic inflammatory gastrointestinal disease which often affects young people and persists through life. It is estimated that CD afflicts more than 500,000 persons in the United States. The prevalence of CD appears to be increasing in the US and other parts of the world. Although it has been well established that *M. avium* subsp. *paratuberculosis* is a cause of Johne's disease in cattle, goats, sheep and other ruminants, a role for this mycobacterium in the pathogenesis of CD remains to be determined.

☆Use of trade names is for identification only and does not constitute endorsement by the US Department of Health and Human Services, the Public Health Services, or the Centers for Disease Control and Prevention.

*Corresponding author. Tel.: +404-639-1474; fax: +404-639-1287.

E-mail address: tms1@cdc.gov (T.M. Shinnick).

1472-9792/03/$ - see front matter Published by Elsevier Science Ltd.
doi:10.1016/S1472-9792(03)00052-0

Efforts to combat mycobacterial diseases are hindered by an inadequate understanding of their underlying pathogenesis and physiology. Culture of mycobacterial pathogens from patients is the standard method for diagnosing mycobacteria-caused diseases. However, the slow growth of these organisms often results in a 3–6 week delay before culture data for *M. tuberculosis* are available and 3–6 months for *M. avium* subsp. *paratuberculosis*. In addition, the recovery of colony-forming units (CFUs) has often been less than anticipated from microscopic observations of acid-fast bacilli. This raises the possibility that dormant or non-replicating cells may be present. And such cells may be viable but not able to form colonies on plates or grow in liquid media.

Studies on the recovery of *Micrococcus luteus* bacteria from a non-replicating phase led to the discovery of a resuscitation promoting factor (Rpf) which can promote the recovery of dormant bacteria. Early studies demonstrated that supernatant from spent medium of *M. luteus* cultures increased the number of viable cells recovered from dormant *M. luteus* cultures.[6–8] The resuscitation factor is secreted into culture medium when the *M. luteus* bacteria enter stationary phase.[9,10] The gene encoding the *M. luteus* Rpf has been cloned and expressed in *E. coli*.[10] The addition of purified Rpf to liquid medium at picomolar concentrations greatly increased the number of viable cells that could be recovered from stationary-phase cultures and shortened the lag-phase.[10,11] Purified Rpf from *M. luteus* can also shorten lag-phase and increase the recovery of *M. tuberculosis*, *M. avium* and other mycobacteria from prolonged stationary cultures.[10–13] Interestingly, the *M. luteus* RPF shares homology with several mycobacterial open reading frames (ORFs).[10,14]

Previous studies have raised the possibility of Rpfs in mycobacteria. Sun and Zhang[15] showed that the addition of supernatant from early stationary cultures of *M. tuberculosis* H37Ra could increase the viability of aged cultures. The resuscitation activity disappeared when the supernatant was treated with heat and acid. An 8-kDa protein was subsequently recovered from culture supernatants that had resuscitation activity.[16] Synthetic peptides derived from this protein could resuscitate dormant bacilli from a 1-year-old culture that were unable to form colonies when plated directly onto solid media. Recently, five genes encoding Rpf homologs from *M. tuberculosis* were cloned and expressed in *E. coli*, and the products of each of the five *rpf* genes were shown to have resuscitation activity and to stimulate bacterial growth.[14]

In the study reported here, we cloned and expressed one *Rpf*-like gene (*mptb*) from *M. avium* subsp. *paratuberculosis* and one (*Rv1009*) from *M. tuberculosis*. These gene products stimulated the growth of mycobacteria and enhanced the recovery of replicating cells from non-replicating phases. Overall, the data indicate that the product of the *M. avium* subsp. *paratuberculosis rpf*-like gene has resuscitation activity and confirm the recent report that *M. tuberculosis* Rv1009 has resuscitation activity.[14]

Material and methods

Strains and media

M. tuberculosis H37Rv, *M. avium* subsp. *paratuberculosis* ATCC43015, and *M. smegmatis 607* bacteria were routinely maintained in Middlebrook 7H9 medium containing 0.05% Tween80 and ADC (albumin-dextrose-catalase) enrichment (Becton Dickinson and Company, Sparks, MD) unless otherwise noted. *M. avium* subsp. *paratuberculosis* cultures were supplemented with 2 mg of ferric mycobactin J (Allied Monitor Inc., Fayette, MO) per liter. Mycobacteria from cultures varying in age from 1 to 4 months were used for growth and resuscitation experiments. *Esherichia coli* bacteria were routinely maintained in Luria-Bertaini (LB) medium.[17] *E. coli* strain DH5α (Life Technologies Inc., Rockville, MD) was used for DNA manipulation and *E. coli* strain BL21(DE3) (Novagen, Madison, CA) was used for protein expression. Sauton medium was prepared as described elsewhere.[18]

Construction of plasmids

Plasmid DNA preparation, restriction endonuclease analysis and ligations were carried out by standard methods described in Sambrook et al.[17] Polymerase chain reaction (PCR) amplifications were performed by using Pfu DNA polymerase (Stratagene, La Jolla, CA) according to manufacturer's instructions. Oligonucleotides were synthesized by the CDC core facility. A 660-bp product (contig 1289, as of May 5, 2002; TIGR Microbial Database) was amplified from *M. avium* subsp. *paratuberculosis* genomic DNA using primers RPF/PTF7 5′-CGCATATGGGCCAGGCGGCCGCGGCCACCGACGGC and RPF/PTBamH1 5′- ACGGATCCTATTAGGCGCTGGGTGCGGGCTGCAC, which has a *BamHI* site at the end. Each PCR reaction was prepared in a total volume of 50 μl including 2 μl dimethyl sulfoxide (DMSO), 8 μl of 1.25 mM dNTP (Amersham Biosciences, Piscataway, NJ), 1 μl of each primer at 20 μM, 2 μl

of DNA (equaling 25–250 ng), 5 μl of 10× Pfu buffer, 1 μl of Pfu enzyme, and 30 μl of H_2O. PCR reactions were performed in a Gene AMP PCR System 9700 (PE Applied Biosystems, Foster City, CA) with reaction cycle: an initial denaturation step of 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 30 s at 60°C, and 1 min 72°C. After amplification, poly(A) overhangs were generated by adding 1 μM of dATP (Amersham Biosciences) and 1 μl of Taq polymerase (PE Applied Biosystems) and by incubating at 72°C for 15 min. The 660-bp amplicon was cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) to generate pMIN26. The nucleotide sequence of the cloned insert was determined using a CEQ 2000 DNA sequencer and the dye terminator cycle sequencing kit (Beckman Coulter, Fullerton, CA). The gene was subcloned into the pET19b expression vector (Novagen) utilizing *NdeI/BamHI* restriction sites to generate pMIN27. pMIN27 was transformed into *E. coli* BL21(DE3) (Novagen) for protein expression studies.

One *M. tuberculosis rpf* gene (*Rv1009*, Acc#e2052146) was amplified from genomic DNA using primers RPFTB3, 5′-CGCATATGGCATG-CAAAACGGTGACGTTGACCGTC-3′, and RPFTBR4, 5′-CGCTCGAGGCGCGCACCCGCTCGTGCAGCACATAC-3′, as described above. The amplicon was cloned into pCR2.1-TOPO (Invitrogen) to generate pMIN2. The 1 kb *NdeI/XhoI* fragment of pMIN2 was cloned into pET21a to generate pMIN9.

Expression and purification of Rpf

The *E. coli* BL21(DE3) strains carrying pMIN9 and pMIN27 were grown in LB medium containing 100 mg of ampicillin (Sigma, St. Louis, MO) per liter overnight at 37°C. The overnight cultures were diluted 1:100 into 1 l of fresh LB-ampicillin medium. When the culture reached mid-log phase ($OD_{600} = 0.5$), expression of the cloned inserts was induced by addition of isopropyl-1thiol-(d)-galactopyranoside (IPTG) (Invitrogen) to a final concentration of 1 mM. After incubation at 37°C for 4–5 h, the bacteria were harvested by centrifugation at 5000*g* for 10 min at 4°C. Cells were lysed by sonication in 50 mM Tris-HCl (pH7.5) containing 1 mM phenylmethysulfony fluoride (Sigma). The sonicate was centrifuged at 7000*g* for 10 min and the soluble fraction was retained for protein purification.

Purification of the His-tagged proteins was performed according to the manufacturer's instructions (Novogen). Briefly, the soluble fraction was applied to a His-tag column (Novogen) previously equilibrated with 1xbinding buffer. The column was washed with 8 volumes of 1xbinding buffer and 5 volumes of 1xwashing buffer. The protein was eluted with 1xelution buffer. The peak fractions were pooled and dialyzed against 10 mM Tris-HCl buffer (pH 7.5) containing 20% glycerol. The purity of protein was determined on an SDS-PAGE gel.[17] The Rpf solution was sterilized using a 0.22-μm Millex®-GV filter (Millipore, Bedford, MA), aliquoted and stored at −80°C. Protein concentration was determined using the BIO-RAD DC protein assay (BIO-RAD, Hercules, CA).

Growth measurement

To prepare bacterial suspensions, bacteria were harvested by centrifugation, washed 1 or 2 times in Sauton medium, and resuspended in Sauton medium. To disperse clumps of mycobacteria, 3-mm glass beads (Kimble Glass Inc, Vineland, NJ) were added (about one-fifth volume) and the cell suspensions with glass beads vortexed for 1 min. The cells were diluted and appropriate dilutions inoculated into medium. Mycobacterial growth was assayed either by turbidity (OD_{600}) measurements or by counting CFUs on Sauton agar plates.

Bacterial counts

The bacterial suspension for total cell counts was prepared as described above. The bacteria were counted microscopically with a Petroff Hausser Counting Chamber (Horsham, PA).

MPN assay

Most probable number (MPN) assays were performed using a 3-month-old culture of *M. tuberculosis* bacteria. The mycobacteria were recovered by centrifugation, the supernatant was discarded, and the cells were resuspended in Sauton media. The suspensions were treated by vortexing with 3-mm glass beads to disperse clumps as described above. The bacterial suspensions were serially diluted such that only a few viable cells were present and portions added to 10 replicate tubes containing Sauton medium. Rpf was added to 5 of the 10 tubes; the 5 remaining tubes without Rpf served as the control. After 40 days of incubation at 37°C, the number of tubes with visible growth was recorded and MPN values were calculated based on the Meynell's table.[19]

BACTEC culture assay

Suspensions of *M. tuberculosis* bacteria were prepared from 1- to 3-month-old cultures as

described above. Bacteria were diluted in Middlebrook 7H9 medium, and 0.4 ml of the dilution (approximately 10^2–10^4 cells/ml) was inoculated into BACTEC® 12B mycobacterial culture vials (Becton Dickinson and Company). Rpf-tb (final concentrations, 16, 64, 256 pM and 1 nM and 5 nM) was added to the cultures, which were then incubated at 37°C without aeration. The growth of the culture was measured daily with the BACTEC-TB 460 system (Becton Dickinson and Company).

Results

Identification of mycobacterial ORFs that are homologous to *M. luteus* Rpf

The available genome sequences of several *Mycobacterium* species were searched to identify gene products homologous to the Rpf of *M. luteus*. The search identified 19 mycobacterial ORFs which shared significant homology with the *M. luteus* RPF: 5 ORFs of *M. tuberculosis* (Rv0867c, Rv1009, Rv1884c, Rv2389c Rv2450c), 3 ORFs of *M. leprae* (ML0250, ML2030, ML2151), 5 ORFs of *M. bovis*, 2 ORFs of *M. smegmatis* (genome unfinished), 2 ORFs of *M. avium* and 2 ORFs of *M. avium* subsp. *paratuberculosis* (genome unfinished). Figure 1 shows partial amino acid alignments of putative mycobacterial Rpfs and the *M. luteus* Rpf. In addition to sharing the conserved domains with the *M. luteus* Rpf, the mycobacterial ORFs share significant homology with each other. For example, Rv0867c shares 63% identity in a 200 amino acid overlap with ML2151 and 70% identity in a 257 amino acid overlap with Av27 (contig 27) and Mptb (contig 1289, as of May 5, 2002; TIGR Microbial Database). Rv1009 shares 43% homology with Rv2450c in a 147 amino acid overlap and Rv1884c shares 55.4% identity with Rv2389c in a 101 amino acid overlap.

In the genome annotations, the functions of these mycobacterial gene products are not assigned and they are classified as conserved hypothetical proteins. Rpf from *M. luteus* is a secreted protein.[12] Analysis of the mycobacterial Rpf-like products using the SMART program (http://smart.embl-heidelberg.de/) indicates that each has a hydrophobic amino acid cluster indicative of a signal sequence or transmembrane helix. This suggests these proteins might be found in an extracytoplasmic (Rv1009) or extracellular location (Rv0867c, Rv1884c, Rv2389c, Rv2450c, Av27, and Mptb). Rv1009 also contains a prokaryotic membrane lipoprotein lipid attachment site.

Expression and purification of recombinant mycobacterial Rpf

To investigate the function of the *M. avium* subsp. *paratuberculosis* Rpf homologue, a 0.66-kb DNA

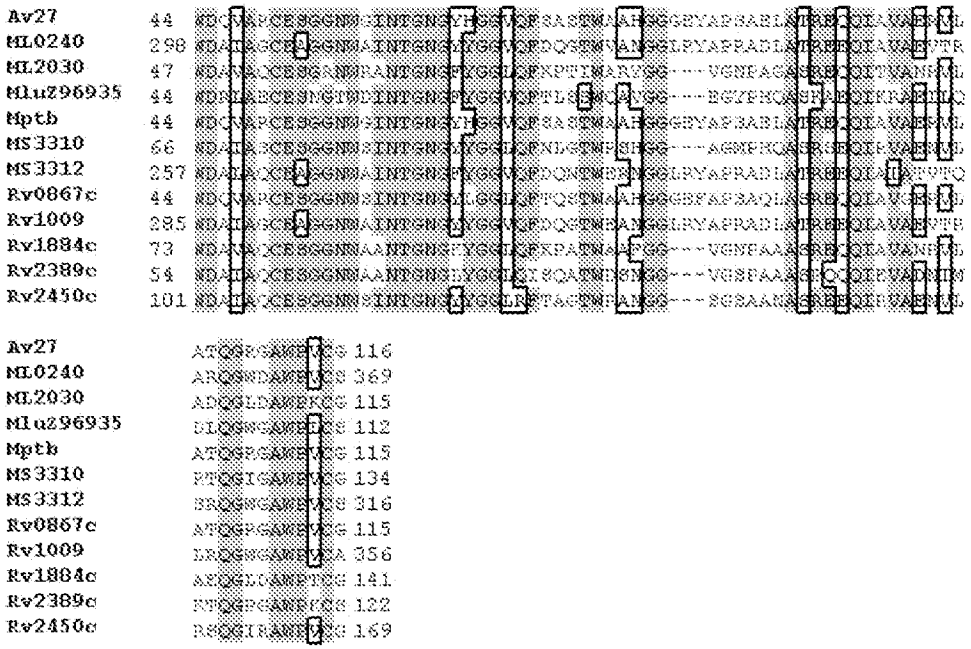

Figure 1 Partial amino acid sequence alignments of putative Rpfs. MluZ96935 is the *M. luteus* Rpf. Rv0867c, Rv1009, Rv1884c, Rv2450c are predicted proteins from *M. tuberculosis*. ML0240 and ML2030 are from *M. leprae*. Av27 is from *M. avium* contig27. Mptb is from *M. avium* subsp. *paratuberculosis* contig1289. MS3310 and MS3312 are from *M. smegmatis*. Black letters with shadow indicate identical residues; boxed letters indicate similar residues.

fragment excluding the predicted secretion signal sequence of the protein was PCR-amplified from genomic DNA using primers RPF/PTF7 and RPF/PTBAMHI. The identity of the PCR product was confirmed by DNA sequencing and it was subcloned into pET19b for protein expression. Using the same strategy, a 1-kb DNA fragment of one of the *M. tuberculosis rpf* genes (*Rv1009*) without its secretion signal sequence was PCR amplified from *M. tuberculosis* genomic DNA and cloned into pET21a for expression. Overexpression of the two cloned genes in *E. coli* produced soluble proteins which appeared as distinct bands on SDS-PAGE (Fig. 2). The expressed proteins were purified by His-tag column chromatography. The size of the recombinant *Rv1009* gene product was 40 kDa as expected. But the size of the recombinant *M. avium* subsp. *paratuberculosis* gene product observed on the polyacrylamide gel was 43 kDa, significantly larger than the predicated size (28 kDa). However, nucleotide sequencing of the cloned DNA and protein sequencing of the amino terminus of the recombinant protein indicated the expressed protein had the correct amino acid sequences. The unexpected apparent size may be related to the high proline (16.8%), alanine (19.5%), and glycine (12%) content of the predicted protein. Following convention, the products of *M. avium* subsp. *paratuberculosis* gene and *M. tuberculosis* gene *Rv1009* were designated as Rpf-mptb and Rpf-tb, respectively. Attempts to express *M. tuberculosis* genes *Rv0867c*, *Rv2389c* and *M. avium Av27* using a similar strategy were unsuccessful. Hence, our analysis focused on Rpf-mptb and Rpf-tb.

Effect of Rpfs on the growth of mycobacteria

To generate mycobacteria that were in a viable but non-replicating phase, cultures that had been maintained for greater than 1 month were used. The quiescent mycobacteria were harvested from such cultures, washed with Sauton media, and resuspended ($\sim 10^2$ bacteria/ml) in Sauton medium with or without Rpf. The final concentration of the recombinant Rpfs varied from 8 to 512 pM. Bacterial growth was followed by optical density.

The Rpf-mptb significantly shortened the lag phase and enhanced the growth of the quiescent *M. avium* subsp. *paratuberculosis* bacteria in a dose-dependent manner (Fig. 3a). The optimal concentrations of Rpf-mptb for growth promotion were between 8 and 128 pM. There was a decrease in the stimulation of growth at the 512-pM concentration. Similarly, the Rpf-tb significantly shortened the lag phase and enhanced the growth of the quiescent *M. tuberculosis* bacteria in a dose-dependent manner (Fig. 3b). The optimal concentrations of Rpf-tb for growth promotion were between 32 and 128 pM. In contrast to the

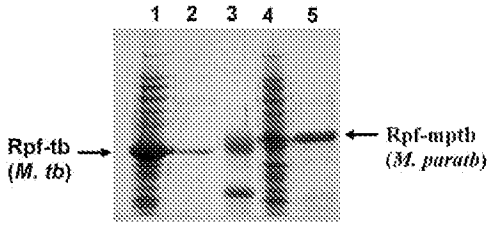

Figure 2 SDS-PAGE analysis of recombinant mycobacterial Rpfs. Lane 1, whole-cell lysate from *E. coli* expressing the *Rv1009* gene; lane 2, purified recombinant product (Rpf-tb); lane 3, molecular size markers, 43 kDa (top band), 38 kDa (lower band); lane 4, whole-cell lysate of *E. coli* expressing the *Mptb* gene; lane 5, purified recombinant Rpf-mptb.

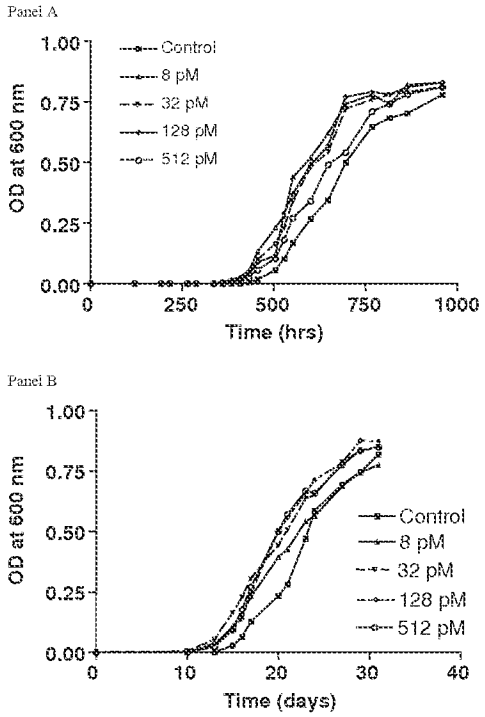

Figure 3 Effect of Rpfs on the growth of mycobacteria. Mycobacteria were harvested by centrifugation, washed once, and resuspended in Sauton medium.[18] About 10^2 cells/ml were inoculated into Sauton medium in the presence or absence of Rpf and the OD600 of the cultures were measured daily. Panel A: Effect of Rpf-mptb (■: 0, ▲: 8, ▼: 32, ♦: 128, or ○: 512 pM) on the growth of 2-month-old *M. avium* subsp. *paratuberculosis*, Panel B: Effect of Rpf-tb (■: 0, ▲: 8, ▼: 32, ♦: 128, or ○: 512 pM) on the growth of 1-month-old *M. tuberculosis* cells.

Table 1 Effect of Rpfs on the growth of *M. tuberculosis* H37Rv bacteria.

	Days	No Rpf (CFU/ml)	+ Rpf-tb (CFU/ml)	+ Rpf-mptb (CFU/ml)	+ Rpf-tb + Rpf-mptb (CFU/ml)
1-month-old culture	0	1.2×10^4	1.2×10^4	1.2×10^4	1.2×10^4
	6	4.9×10^4	5.2×10^5	6.9×10^5	6.4×10^5
	10	3.1×10^5	2.4×10^7	3.9×10^7	4.1×10^7
4-month-old culture	0	3.7×10^2	3.7×10^2	3.7×10^2	3.7×10^2
	6	1.3×10^3	1.8×10^4	2.4×10^4	1.7×10^4
	10	6.5×10^3	7.4×10^5	8.3×10^5	5.9×10^5

Rpf: Resuscitation promoting factor; CFU: colony-forming unit

Rpf-mptb results, there was not a decrease in the stimulation of growth at the 512-pM concentration but rather it leveled off.

To measure the effect on cell growth at earlier time points than possible with optical density measurements, *M. tuberculosis* H37Rv bacteria were harvested from a 1-month-old culture and used to inoculate Sauton media containing 128 pM Rpf-tb, 128 pM Rpf-mptb, 128 pM Rpf-tb and 128 pM Rpf-mptb, or no Rpf. The number of CFUs was determined at 0, 6, and 10 days post-inoculation (Table 1). At 6 days post-inoculation, 10- to 14-fold more CFUs were recovered from the cultures containing either of the Rpfs. At 10 days post-inoculation, 76- to 132-fold more CFUs were recovered from the cultures containing either of the Rpfs. The recovery of CFUs from the cultures containing the mixture of Rpfs was not significantly different than the recovery from the cultures with the individual Rpfs. Similar results were obtained when bacilli from a 4-month-old culture of *M. tuberculosis* H37Rv were used (Table 1).

Rpf activity can also be measured using washed *M. smegmatis* bacteria because when washed *M. smegmatis* cells are used to inoculate Sauton medium, there is no significant increase in CFUs during a 10-day incubation without the addition of Rpf to the medium.[11] The use of a relatively poor medium and low inoculum is required to observe the effect: that is, washed *M. smegmatis* cells will grow well in the absence of Rpf if a large inoculum is used ($>10^2$ cells/ml) or if a rich medium such as Middlebrook 7H9 is used (data not shown). As shown in Fig. 4, extensively washed *M. smegmatis* bacteria from an early stationary phase culture were used to inoculate Sauton media ($<10^2$ bacteria/ml) with or without Rpf-tb and growth followed by OD600 measurements. No measurable growth was detected in the cultures without Rpf-tb. Growth of the washed *M. smegmatis* cells was strongly stimulated in a dose-dependent manner when Rpf-tb was added at concentrations from 250 pM to 5 nM.

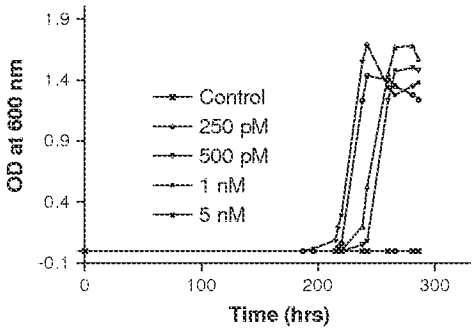

Figure 4 Rpf-dependent growth of *M. smegmatis* 607. Bacteria from early stationary phase were recovered, washed in Sauton medium[18] 5 times, and used to inoculate a 50-ml flask containing 20 ml Sauton media with or without Rpf. The cultures were incubated at 37°C and OD600 measured daily. ■: Control without Rpf; ▲: 250 pM; ▼: 500 pM; ◆: 1 nM; and : 5 nM Rpf-tb.

MPN assay

To determine if the Rpfs were acting to reduce the lag phase or to 'resuscitate' dormant cells, MPN assays were done to determine the number of viable bacteria recoverable from a culture.[11–13] In an MPN assay, bacteria are diluted to such an extent that portions containing only one or a few viable cells are used to inoculate a set of replicate cultures and the number of test cultures in which bacteria grow is used to calculate the number of viable cells in the original sample. To measure the resuscitation activity of the mycobacterial Rpfs, an MPN assay was done using a 3-month-old *M. tuberculosis* H37Rv bacterium (Table 2). As is typically observed for old cultures of tubercle bacilli, there are many more bacteria in the culture

Table 2 Resuscitation activity of Rpfs on a of 3-month-old *M. tuberculosis* H37Rv culture.

	Cells/ml	Recovery (%)
Total cell count	6.9×10^7	—
CFU counts	1.5×10^5	0.2
Sauton – no Rpf	1.1×10^6	1.4
Sauton + Rpf-tb	9.2×10^6	13
Sauton + Rpf-mptb	1.6×10^7	23

Rpf: Resuscitation promoting factor; CFU: colony-forming unit.

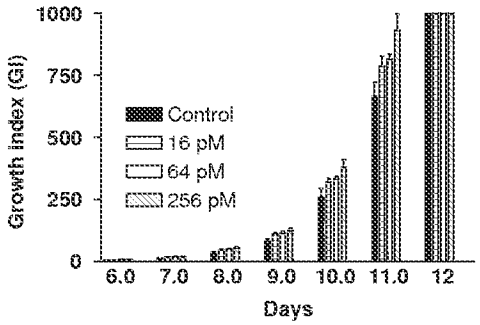

Figure 5 Effect of Rpf-tb on the growth of *M. tuberculosis* in BACTEC cultures (Becton Dickinson and Company, Sparks, MD). Bacteria were inoculated into BACTEC® 12B culture vials in the presence of different concentrations of Rpf-tb, and the growth of the cultures was determined daily by the BACTEC-TB 460 system. The results represent the average of triplicate experiments.

as determined by microscopy than there are bacteria capable of forming colonies when portions are plated directly onto solid media (6.9×10^7 bacteria vs 1.5×10^5 CFU; Table 2). By MPN assay, 1.1×10^6 viable cells (1.4% of the bacteria) could be recovered by inoculating Sauton medium. The addition of either the Rpf-tb (128 pM) or the Rpf-mptb (128 pM) resulted in the recovery of 8- to 15-fold more viable cells from the 3-month-old culture. The addition of either Rpf to the rich Middlebrook 7H9 medium did not enhance recovery (data not shown).

BACTEC culture assay

The BACTEC 12B mycobacterial culture system was employed to further test the effect of Rpf-tb on the mycobacterial growth. The BACTEC 12B mycobacterial medium is an enriched Middlebrook 7H9 broth. In this system, the growth of mycobacteria is measured by the release of $^{14}CO_2$ from ^{14}C-labeled palmitic acid. The addition of Rpf-tb to BACTEC vials inoculated with bacteria from a 3-month-old culture of *M. tuberculosis* H37Rv only slightly increased the growth of the bacteria in a dose-dependent manner (Fig. 5), similar to the results in the studies with rich media described above.

Discussion

Proteins with sequence homology to Rpfs are widely distributed among member of GC-rich Gram-positive bacterial genera such as *Mycobacterium*, *Micrococcus*, *Streptomyces*, and *Corynebacterium*.[10] In database searches, 19 homologs of the *M. luteus* Rpf were identified in the genomes of several mycobacteria. For example, 5 Rpf homologs were found in the *M. tuberculosis* genome. The role of the individual Rpf proteins and reasons for redundancy are not yet known.

Initially, we chose 5 putative *rpf* genes (*Rv1009*, *Rv0867c*, *Rv2389c*, *Av27* and *Mptb*) for study. We chose these genes to reflect the variation in the Rpf homologs. Rv1009 (362 residues) has a longer N-terminal sequences than does the *M. luteus* Rpf, *Rv0867c* has a longer C-terminal sequence, and *Rv2389c* has shorter N- and C-terminal sequences. Av27 and Mptb were chosen as representatives from *M. avium* and *M. avium* subsp. *paratuberculosis*. Expression of *Rv0867c*, *Rv2389c* and *Av27* was poor in *E. coli* in our experiments. Similarly, Mukamolova et al.[14] reported obtaining only weak expression of *Rv0867c* and *Rv2389c* in their experiments.

In addition to the amino acid sequence homologies, three lines of evidence indicate that the products of the *Mptb* and *Rv1009* genes have resuscitation activity: the proteins can (i) shorten the lag time and promote the growth of bacteria cultured from a quiescent or stationary phase, (ii) increase the number of bacteria capable of growing that can be recovered from a quiescent or stationary phase culture, and (iii) support the growth of *M. smegmatis* bacteria that have been extensively washed and inoculated into Sauton medium. Our results with the Rv1009 Rpf confirm the demonstration of the resuscitation activity of Rv1009 by Mukamolova et al.[15] The ~10-fold increase in viable bacteria recovered from an *M. tuberculosis* 3-month-old culture is consistent with the effects seen in other systems with other resuscitation factors.[10–12,14]

The rapid and sensitive detection of mycobacteria in clinical specimens is important for the

laboratory confirmation of an infection. Because clinical specimens may contain a mixture of actively growing and quiescent mycobacteria, our initial interest in Rpfs arose from the possibility of improving the recovery of mycobacteria from clinical specimens. For example, studies aimed at recovering *M. avium* subsp. *paratuberculosis* bacteria from tissue specimens from Crohn's disease patients have had limited success and visible colonies take 6–18 months to appear,[3,4] if at all. Recovery of tubercle bacilli from sputum specimens can take as long as 8–10 weeks. In a minimal medium such as Sauton medium, both of the mycobacterial Rpfs could increase the recovery of mycobacteria by 10-fold or more. Unfortunately, very little, if any, improvement in recovery could be detected when rich media such as Middlebrook 7H9 or BACTEC media were used. This result may not be entirely unexpected because Middlebrook 7H9 media and the BACTEC media were developed to optimize the recovery of tubercle bacilli from clinical specimens.[20–23] Further studies are needed to determine if the mycobacterial Rpfs can improve the recovery of *M. tuberculosis* or *M. avium* subsp. *paratuberculosis* bacteria from clinical samples.

Acknowledgements

This work is supported by funding from the Chronic Diseases Working group of the Emerging Infectious Diseases Program of the National Center for Infectious Diseases. We thank Berrin Gencer and David Sikes for technical assistance. We thank the Biotechnology Core Facility of the Scientific Resources Program of the National Center for Infectious Diseases for providing oligonucleotides primers and protein sequencing.

References

1. Centers for Disease Control and Prevention. Reported cases of tuberculosis in the United States, 2000. CDC, Division of Tuberculosis Elimination, Atlanta, GA. 2001.
2. World Health Organization report 2002, Global tuberculosis control: surveillance, planning, financing. WHO report 2002, Geneva, Switzerland. WHO/CDS/TB/2002.295.
3. Chiodini RJ, Van Kruiningen HJ, Merkkal RS. Ruminant paratuberculosis (Johne's disease): the current status and future prospects. *Cornell Vet* 1984;**74**:218–62.
4. Collins MT, Lisby G, Moser G, Chicks D, Christensen S, Reichelderfer M, Hoiby N, Harms BA, Thomsen OO, Skibsted U, Binder V. Results of multiple diagnostic tests for *Mycobacterium avium* subsp. paratuberculosis in patients with inflammatory bowel disease and in controls. *J Clin Microbiol* 2000;**38**:4373–81.
5. McFadden JJ, Butcher PD, Chiodini R, Hermon-Taylor J. Crohn's disease-isolated mycobacteria are identical to *Mycobacterium paratuberculosis*, as determined by DNA probes that distinguish between mycobacterial species. *J Clin Microbiol* 1987;**25**:796–801.
6. Kaprelyants AS, Kell DB. Dormancy in stationary-phase cultures of *Micrococcus luteus*: flow cytometric analysis of starvation and resuscitation. *Appl Environ Microbiol* 1993; **59**:3187–96.
7. Kaprelyants A, Mukamolova GV, Davey HM, Kell DB. Quantitative analysis of the physiological heterogeneity within starved cultured of *Micrococcus luteus* by flow cytometry and cell sorting. *Appl Environ Microbiol* 1996; **62**:1311–6.
8. Votyakova TV, Kaprelyants AS, Kell DB. Influence of viable cells on resuscitation of dormant cells in *Micrococcus luteus* cultures held in an extended stationary phase: the population effect. *Appl Environ Microbiol* 1994;**60**:3284–91.
9. Kaprelyants A, Mukamolova G V, Kell DB. Estimation of dormant *Micrococcus luteus* cells by penicillin lysis and by resuscitation in cell-free spent medium medium at high dilution. *FEMS Microbiol Lett* 1994;**115**:347–52.
10. Mukamolova GV, Kaprelyants AS, Young DI, Young M, Kell DB. A bacterial cytokine. *Proc Natl Acad Sci USA* 1998;**95**: 8916–21.
11. Mukamolova GV, Kormer SS, Kell DB, Kaprelyants AS. Stimulation of the multiplication of *Micrococcus luteus* by an autocrine growth factor. *Arch Microbiol* 1999;**172**:9–14.
12. Shleeva MO, Bagramyan K, Telkov MV, Mukamolova GV, Young M, Kell DB, Kaprelyants AS. Formation and resuscitation of "non culturable" cells of *Rhodococcus rhodochrous* and *Mycobacterium tuberculosis* in prolonged station phase. *Microbiology* 2002;**148**:1581–91.
13. Biketov S, Mukamolova GV, Potapov V, Gilenkov E, Vostroknutov G, Kell DB, Young M, Kaprelyants AS. Culturability of *Mycobacterium tuberculosis* cells isolated from murine macrophages: a bacterial growth factor promotes recovery. *FEMS Immunol Med Microbiol* 2000;**29**:233–40.
14. Mukamolova G, Turapov OA, Young DI, Kaprelyants AS, Kell DB, Young M. A family of autocrine growth factors in *Mycobacterium tuberculosis*. *Mol Microbiol* 2002;**46**: 623–35.
15. Sun Z, Zhang Y. Spent culture supernatant of *Mycobacterium tuberculosis* H37Ra improves viability of aged cultures of this strain and allows small inocula to initiate growth. *J Bacteriol* 1999;**181**:7626–8.
16. Zhang Y, Yang Y, Woods A, Cotter R J, Sun Z. Resuscitation of dormant *Mycobacterium tuberculosis* by phospholipids or specific peptides. *Biochem Biophys Res Commun* 2001;**8**:542–7.
17. Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*, 2nd ed.. Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989.
18. Allen BW. Mycobacteria: general culture methodology and safety considerations. In: Parish T, Stoker NG, editors. Mycobacteria protocols, methods in molecular biology, vol. 101. Totowa, NJ: Humana Press, 1998. p. 10.
19. Meynell GG, Meynell E. Theory and practice in experimental bacteriology. Cambridge, Cambridge University Press, 1970. p. 219–36.
20. Anargyros PD, Astill DSJ, Sim IS. Comparison of improved BACTEC and Lowenstein-Jensen medium for culture of mycobacteria from clinical specimens. *J Clin Microbiol* 1990;**28**:1288–91.
21. Kirihara JM, Hillier SL, Coyle MB. Improved detection times for *Mycobacterium avium* complex and *Mycobacterial*

tuberculosis with the BACTEC radiometric system. *J Clin Microbiol* 1985;**22**:841–5.

22. Park CH, Hixon DL, Ferguson CB, Hall SL, Risheim CC, Cook CB. Rapid recovery of mycobacteria from clinical specimens using automated radiometric technique. *Am J Clin Pathol* 1984;**81**:341–5.
23. Tokars JL, Rudnick JR, Kroc K, Manangan L, Pugliese G, Huebner RE, Chan J, Jarvis WR. US hospital mycobacteriology laboratories: status and comparison with state public health department laboratories. *J Clin Microbiol* 1996; **34**:680–5.